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Changes in glucose 1,6-bisphosphate content in rat skeletal muscle during contraction

Anna M. BASSOLS, José CARRERAS and Roser CUSSÓ*

Departmento de Bioquímica, Facultad de Medicina, Universidad de Barcelona, Zona Universitaria–Pedralbes, 08028 Barcelona, Spain

Glucose 1,6-bisphosphate, fructose 2,6-bisphosphate, glycogen, lactate and other glycolytic metabolites were measured in rat gastrocnemius muscle, which was electrically stimulated *in situ* via the sciatic nerve. Both the frequency and the duration of stimulation were varied to obtain different rates of glycolysis. There was no apparent relationship between fructose 2,6-bisphosphate content and lactate accumulation in contracting muscle. In contrast, glucose 1,6-bisphosphate content increased with lactate concentration during contraction. It is suggested that the increase in glucose 1,6-bisphosphate could play a role in phosphofructokinase stimulation and in the activation of the glycolytic flux during muscle contraction.

INTRODUCTION

It is generally accepted that the increase in the rate of glycolysis which takes place during muscle contraction involves an activation of phosphofructokinase, although the mechanism of this activation is not fully understood (Newsholme & Start, 1973). Phosphofructokinase is a highly modulated enzyme, whose activity responds to a variety of metabolic signals (Sols *et al.*, 1981). Mammalian phosphofructokinases are susceptible to allosteric regulation by many metabolites, including substrates and products, in a pH-dependent way (Uyeda, 1979). Both the liver and the muscle enzymes have been found to be susceptible to inactivation by phosphorylation *in vivo* (Brand & Söling, 1975; Hofer & Sørensen-Zinganke, 1979). The kinetic behaviour and the sensitivity to allosteric effectors of animal phosphofructokinases are markedly affected by the enzyme concentration and aggregation state (Reinhart, 1980; Boscá *et al.*, 1985). It has been shown that calmodulin, by changing the aggregation state and the conformation of muscle phosphofructokinase, may induce a hysteretic inactivation/reactivation of the enzyme in a way essentially dependent on metabolic conditions (Mayr, 1984*a*, *b*).

In addition to the product Fru-1,6-P_2, two other sugar bisphosphates, Fru-2,6-P_2 and Glu-1,6-P_2, have been described as modulators of phosphofructokinase. The three hexose bisphosphates may act as allosteric activators of both liver and muscle phosphofructokinase (Sols *et al.*, 1981; Hers & Van Schaftingen, 1982; Beitner, 1984). They enhance the rate of phosphofructokinase phosphorylation by cyclic AMP-dependent protein kinase (Kemp & Foe, 1983), and may influence the hysteretic calmodulin-mediated inactivation/reactivation of the muscle enzyme (Mayr, 1984*b*). Low fractional saturation of hexose bisphosphate binding sites is a prerequisite for inactivation to occur, and high concentrations of sugar bisphosphates are additional reactivation factors (Mayr, 1984*b*).

It has been suggested that Fru-2,6-P_2 plays a key role in the stimulation of muscle phosphofructokinase and in the activation of the glycolytic flux during initiation of insect flight (Storey, 1983). But the role of Fru-2,6-P_2 in the stimulation of glycolysis during contraction in mammalian muscle is unclear (Hue *et al.*, 1982; Minatogawa & Hue, 1984). No relationship was found between Fru-2,6-P_2 content and the increase in glycolytic flux when rat muscles were electrically stimulated *in situ* (Minatogawa & Hue, 1984).

Glu-1,6-P_2 is a potent regulator of several enzymes involved in carbohydrate metabolism (Beitner, 1984). In muscle, it has been shown to activate (de-inhibit) phosphofructokinase (Hofer & Pette, 1968; Rose & Warms, 1974) and phosphoglucomutase (Leloir *et al.*, 1948), and to inhibit hexokinase (Beitner *et al.*, 1975). The concentration of Glu-1,6-P_2 in cultured muscle tissue varied with the development state. The changes in the concentration of the metabolite were accompanied by changes in the [Fru-1,6-P_2]/[Fru-6-P] ratio, implying an effect on phosphofructokinase activity (Wakelam & Pette, 1982). It has been shown that Glu-1,6-P_2 concentration in muscle changes in several hormonal, physiological and pathological conditions (Beitner, 1984), but Glu-1,6-P_2 contents during contraction have not been studied.

In the present study we have examined the changes in Glu-1,6-P_2 content produced in rat gastrocnemius muscle during contraction, and compared these changes with those of Fru-2,6-P_2 and Fru-1,6-P_2. The muscle was electrically stimulated *in situ* in anaesthetized rats, at different frequencies and for different periods of time, to obtain different rates of glycolysis. The results reported show that Glu-1,6-P_2 content increases concurrently with lactate production during contraction.

MATERIALS AND METHODS

Materials

Enzymes and biochemicals were purchased from either Boehringer or Sigma. Fru-2,6-P_2 was generously given by Dr E. Van Schaftingen. Dowex AG1-X8 (Cl^- form;

Abbreviations used: Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Glu-1,6-P_2, glucose 1, 6-bisphosphate.

* To whom correspondence should be addressed.

200–400 mesh) was from Bio-Rad. Glucose 1-phosphate was freed of Glu-1,6-P_2 as described by Climent *et al.* (1985). All other chemicals were reagent grade.

Methods

Preparation of muscle and electrical stimulation. Experiments were carried out on fed male Sprague–Dawley rats (200–300 g body wt.), which were anaesthetized with thiopental (70 mg/kg, intraperitoneally). The skin from the right hindleg was removed, and the sciatic nerve and the gastrocnemius muscle were exposed and prepared as described by Minatogawa & Hue (1984). The rats were allowed to recover for 20 min, and isometric contractions were then induced by electrical stimulation of the sciatic nerve with a Letica LI 12100 stimulator. The gastrocnemius muscle was stimulated for various periods of time, at 5 V and at frequencies of 5 and 25 Hz. The contracting muscle was frozen *in situ* between stainless-steel clamps precooled in liquid N_2. Controls were treated similarly but not stimulated.

Preparation of extracts and measurements of metabolites. Samples of frozen muscles were powdered in a stainless-steel percussion mortar precooled in liquid N_2, and used for the measurement of metabolites.

For the measurement of Fru-2,6-P_2, about 30 mg of frozen powder was homogenized with a Potter–Elvehjem homogenizer in 10 vol. of 50 mM-NaOH and kept at 90 °C for 10 min. The alkaline extract was neutralized at 0 °C by the addition of 250 mM-sodium acetate, pH 4.0, and the insoluble material was removed by centrifugation at 4 °C for 5 min in an Eppendorf Microfuge. Fru-2,6-P_2 was measured in the supernatant as described by Van Schaftingen *et al.* (1982).

For the measurement of Glu-1,6-P_2 and other metabolites, about 300 mg of frozen powder was homogenized at 0 °C in 5 vol. of 10% (v/v) $HClO_4$ with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 3000 rev./min for 20 min, and the supernatant was neutralized to pH 7 with 3 M-KOH/$KHCO_3$. Metabolites were determined spectrophotometrically with a Kontron Uvikon 810 spectrophotometer by the following enzymic methods: lactate (Gutmann & Wahlefeld, 1974), Fru-1,6-P_2 (Michal & Beutler, 1974), glucose 6-phosphate (Michal, 1984), 2,3-bisphosphoglycerate (Towne *et al.*, 1957) and ATP (Lamprecht & Trautschold, 1974).

The measurement of Glu-1,6-P_2 in tissue extracts as a cofactor of the phosphoglucomutase reaction (Passonneau *et al.*, 1969) presents some difficulties, owing to the large number of metabolites that inhibit this enzyme, especially Fru-1,6-P_2. In order to destroy this metabolite, before assay the samples were heated at 80 °C at basic pH (0.1 M-KOH) for 1 h and then neutralized with 10% $HClO_4$.

Glycogen was determined by the anthrone method (Carroll *et al.*, 1956).

RESULTS

Assessment of the results

To verify the concentration of Glu-1,6-P_2 measured in extracts, samples from heated extracts were applied to a column (0.5 cm × 5 cm) of Dowex AG1-X8 (Cl^- form). The column was first washed with 50 mM-NH_4Cl to elute the monophosphorylated compounds, and then with 250 mM-NH_4Cl to elute the bisphosphorylated compounds. The concentration of Glu-1,6-P_2 found in the eluates was not significantly different from that determined in the original extracts (Table 1).

The recovery of sugar phosphates after chromatography was assessed by applying a mixture of 20 mM-glucose 1-phosphate and 20 mM-Glu-1,6-P_2 to the column. The yields were about 85%.

Influence of blood contamination on Glu-1,6-P_2 concentration in muscle extracts

The concentration of Glu-1,6-P_2 is higher in erythrocytes (80 nmol/g; Passonneau *et al.*, 1969) than in skeletal muscle (20 nmol/g). Therefore, owing to blood contamination of muscle extracts, the changes in blood flux produced during contraction could mask the changes in Glu-1,6-P_2 concentration produced in the muscle. To test this possibility, the gastrocnemius muscle was stimulated at different frequencies for different periods of time, and the concentrations of Glu-1,6-P_2 and 2,3-bisphosphoglycerate were measured in the extracts. Since 2,3-bisphosphoglycerate concentrations are much higher in erythrocytes than in muscle (Tauler *et al.*, 1986), the variations in 2,3-bisphosphoglycerate concentration in the extracts reflect differences in blood contamination. The results obtained (not shown) did not show any correlation between concentrations of 2,3-bisphosphoglycerate and Glu-1,6-P_2 and the length and intensity of contraction. The concentration of Glu-1,6-P_2 in muscle extracts caused by blood contamination was calculated to be 2 nmol/g of tissue.

Changes in metabolite concentrations induced by muscle contraction

The time course of the changes in metabolite concentrations was studied in gastrocnemius muscle stimulated at 5 Hz (sub-tetanic contractions) and at 25 Hz (tetanic contractions).

As shown in Table 2, when the muscle was stimulated for different periods of time at a frequency of 5 Hz, lactate accumulated (average rate 4.5 μmol/min per g).

Table 1. Recovery of glucose 1,6-bisphosphate on a Dowex AG1-X8 column

Samples (100 μl) of the neutralized heated extracts from gastrocnemius muscle subjected to different conditions of stimulation were applied to a Dowex AG1–X8 column, and eluted as described in the text.

Stimulation	Glu-1,6-P_2 applied (nmol)	Glu-1,6-P_2 recovered (nmol)	(%)
None (control)	0.241	0.176	73
	0.150	0.144	96
	0.222	0.171	77
5 Hz, 20 s	0.402	0.326	81
5 Hz, 60 s	0.683	0.533	78
5 Hz, 120 s	0.250	0.242	96
25 Hz, 10 s	0.305	0.278	91
25 Hz, 30 s	0.300	0.293	97
25 Hz, 60 s	0.356	0.329	92

Table 2. Concentrations of metabolites in gastrocnemius muscle stimulated for different periods of time at a frequency of 5 Hz

Results are expressed in μmol/g wet wt. of tissue. Fru-2,6-P_2 and Glu-1,6-P_2 are expressed in nmol/g, and glycogen as μmol of glucose equivalent/g. Values shown are means ± S.E.M. for the numbers of experiments in parentheses: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, for difference from zero time.

	Metabolite concn.			
Time of stimulation ...	0 s	20 s	60 s	120 s
Glu-1,6-P_2	16.25 ± 1.99 (12)	28.12 ± 2.83*** (4)	40.78 ± 7.83** (4)	36.69 ± 4.70*** (5)
Fru-2,6-P_2	0.468 ± 0.03 (10)	0.362 ± 0.06 (4)	0.412 ± 0.05 (4)	0.387 ± 0.04 (5)
Lactate	2.13 ± 0.28 (12)	3.93 ± 0.99** (4)	6.07 ± 0.66*** (4)	10.27 ± 1.12*** (5)
Glycogen	27.1 ± 13.03 (12)	25.3 ± 3.05 (4)	21.0 ± 2.77 (4)	16.2 ± 2.55 (5)
Glucose 6-phosphate	0.32 ± 0.13 (5)	1.67 ± 0.45* (4)	2.04 ± 0.26*** (4)	2.97 ± 0.26*** (5)
Fru-1,6-P_2	0.11 ± 0.04 (12)	0.36 ± 0.28*** (4)	0.28 ± 0.02*** (4)	0.21 ± 0.03** (5)
ATP	4.38 ± 0.61 (11)	3.52 ± 0.19 (4)	3.83 ± 0.14 (4)	3.39 ± 0.12 (5)

Table 3. Concentrations of metabolites in gastrocnemius muscle stimulated for different periods of time at a frequency of 25 Hz

Results are expressed in μmol/g wet wt. of tissue. Fru-2,6-P_2 and Glu-1,6-P_2 are expressed in nmol/g, and glycogen as μmol of glucose equivalent/g. Values shown are means ± S.E.M. for the numbers of experiments in parentheses: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, for difference from zero time.

	Metabolite concn.			
Time of stimulation ...	0 s	10 s	30 s	60 s
Glu-1,6-P_2	16.25 ± 1.99 (12)	26.78 ± 4.46* (3)	32.10 ± 3.83** (5)	25.21 ± 3.07* (4)
Fru-2,6-P_2	0.468 ± 0.03 (10)	0.762 ± 0.07* (3)	0.358 ± 0.05 (5)	0.370 ± 0.06 (4)
Lactate	2.13 ± 0.28 (12)	3.14 ± 0.67* (3)	5.86 ± 0.73*** (5)	7.82 ± 0.49 (4)
Glycogen	27.1 ± 13.03 (12)	24.9 ± 2.55 (3)	22.7 ± 2.05 (5)	15.7 ± 0.55 (4)
Glucose 6-phosphate	0.32 ± 0.13 (5)	0.43 ± 0.08 (3)	2.02 ± 0.18*** (5)	2.77 ± 0.47*** (4)
Fru-1,6-P_2	0.11 ± 0.04 (12)	1.17 ± 0.13*** (3)	0.40 ± 0.06*** (5)	0.32 ± 0.05*** (4)
ATP	4.38 ± 0.61 (11)	4.76 ± 0.26 (3)	4.89 ± 0.53 (5)	4.28 ± 0.06 (4)

The concentration of glucose 6-phosphate increased 9-fold at a relatively constant rate, indicating that the supply of glycolytic substrates exceeded the glycolytic rate (Minatogawa & Hue, 1984). Fru-1,6-P_2 concentration increased up to 4 times the rest values during the first 20 s of stimulation. Glu-1,6-P_2 content increased 3-fold at 1 min, and then remained constant. The concentration of Fru-2,6-P_2 did not change significantly during a 2 min period of stimulation. This is in contrast with the observations of Minatogawa & Hue (1984), who under similar experimental conditions reported a transient increase in Fru-2,6-P_2 concentration.

Table 3 summarizes the changes in metabolite concentrations when gastrocnemius muscles were stimulated at a frequency of 25 Hz. There was a progressive loss of glycogen and a progressive accumulation of lactate, at rates which were higher than those observed with 5 Hz stimulation. The concentration of glucose 6-phosphate increased continuously to reach values that were 9 times the initial rest content, and Glu-1,6-P_2 concentration increased to twice the rest values during the first 30 s of stimulation. As observed in the stimulation at 5 Hz, the change in Fru-1,6-P_2 concentration was biphasic, although the transient increase in the content of the metabolite was more pronounced at the higher frequency of stimulation (15-fold). A similar biphasic pattern has been described by Minatogawa & Hue (1984), who interpreted it as resulting from a

reaction in the further metabolism of Fru-1,6-P_2 (probably the glyceraldehyde-3-phosphate dehydrogenase reaction) being transiently limiting.

DISCUSSION

The physiological importance of the three bisphosphorylated hexoses (Fru-2,6-P_2, Fru-1,6-P_2 and Glu-1,6-P_2) as activators of muscle phosphofructokinase during contraction is controversial.

Although some discrepancies exist between the changes in Fru-2,6-P_2 concentration in the contracting muscle observed by Minatogawa & Hue (1984) and the results reported herein, our findings confirm that there is no clear relationship between Fru-2,6-P_2 content and lactate accumulation during contraction. Our results show that, in contrast with Fru-2,6-P_2, Glu-1,6-P_2 content increases concurrently with the accumulation of lactate. Fru-1,6-P_2 reaches a high concentration in the first moments of contraction, and then it returns to lower values. This biphasic profile has been described by others (Minatogawa & Hue, 1984).

Fru-1,6-P_2, Fru-2,6-P_2 and Glu-1,6-P_2 compete to bind to muscle phosphofructokinase (Foe *et al.*, 1983). The apparent affinity of muscle phosphofructokinase for Glu-1,6-P_2 is about two orders of magnitude smaller than for Fru-2,6-P_2 (Foe *et al.*, 1983). However, muscle Glu-1,6-P_2 content is much greater than Fru-2,6-P_2 content, mainly during contraction (about 100-fold). Glu-1,6-P_2 concentration in contracting muscle (80 μM) becomes one order of magnitude greater than that (7.5 μM) necessary for half-maximal activation of muscle phosphofructokinase (Foe *et al.*, 1983). The Glu-1,6-P_2 concentration in resting muscle (30 μM) is already higher than the K_a of phosphofructokinase, but Glu-1,6-P_2 could be partially bound to enzymes, mainly to phosphoglucomutase and other phosphomutases which use Glu-1,6-P_2 as cofactor (Beitner, 1985; Rose, 1986). Phosphoglucomutase has a very low K_d (19 nM) for the metabolite (Ray & Long, 1976). Nevertheless, as the coenzyme concentration is probably one order of magnitude higher than the phosphoglucomutase concentration on a molar basis (Passonneau *et al.*, 1969), the percentage of bound Glu-1,6-P_2 cannot be very large. No results are available about the other enzymes that are able to bind Glu-1,6-P_2.

It has been calculated that as much as 90% of muscle Fru-1,6-P_2 can be bound to phosphofructokinase and aldolase (Tornheim & Lowenstein, 1976). The situation is different for Fru-2,6-P_2, owing to its low concentration. It has been pointed out (Foe *et al.*, 1983) that, even if fully bound to phosphofructokinase, in rat skeletal muscle Fru-2,6-P_2 is sufficient to bind 8% of the enzyme subunits.

On the other hand, the real K_a of phosphofructokinase for Glu-1,6-P_2 in the presence of other inhibitory metabolites present *in vivo*, such as ATP and citrate, could be higher than that reported in standard conditions. This has been found to occur for Fru-2,6-P_2 (Tornheim, 1985) and for Fru-1,6-P_2 (Tornheim & Lowenstein, 1976). Furthermore, phosphofructokinase activation by Glu-1,6-P_2 could be affected by the concentration of the enzyme. In contrast with liver phosphofructokinase, the muscle isoenzyme is not significantly activated by Fru-2,6-P_2 at physiological concentrations of enzyme, substrates and effectors (Boscá *et al.*, 1985). Therefore the increase in Glu-1,6-P_2 during contraction could play a significant role in the stimulation of phosphofructokinase and in the activation of the glycolytic flux.

Finally, the possibility that an increase in Glu-1,6-P_2 during contraction could enhance phosphofructokinase activity by influencing its aggregation state has to be considered. Both the hysteretic calmodulin-induced inactivation and the calmodulin-mediated reactivation of phosphofructokinase are essentially dependent on environmental conditions (Mayr, 1984*b*). The low concentrations of Glu-1,6-P_2, Fru-2,6-P_2 and Fru-1,6-P_2 in resting muscle would contribute to the Ca^{2+}-dependent inactivation of phosphofructokinase by calmodulin. The increase in Glu-1,6-P_2 produced during contraction, acting in a concerted way with the increase in Fru-1,6-P_2, would contribute to the calmodulin reactivation of phosphofructokinase and increase the glycolytic flux (Mayr, 1984*a*).

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